CHARGE NUMBER: Project 1904

PROJECT TITLE: Tobacco Physiology and Biochemistry

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Objective: To establish the time course and biochemical changes characteristic of tobacco leaves at various stages during senescence.

Status:

I. Enzyme Assays and Associated Methodologies.

In an attempt to demonstrate endopeptidase and/or carboxypeptidase activity in fresh, green, bright (Coker 319) leaf, extractions were prepared at four different pHs (pH 5, 6, 7, and 8). Each extract was also concentrated 10-15 X using an Amicon stirred ultrafiltration cell with a filter molecular weight cutoff of 10,000. When these extracts and their concentrates were tested, no activity was found for either carboxypeptidase A or B; and only the concentrated extracts appeared to have activity in the endopeptidase assay. Although it required a concentration step, this was the first time endopeptidase activity in tobacco extracts had been demonstrated in our lab. A hint of activity for the concentrated extracts was also found when these extracts were screened in an endoproteinase fibrin clot assay marketed by Boehringer Mannheim. As with all previous extracts, leucine aminopeptidase activity was present in all of the extracts tested.

Since we were unable to detect carboxypeptidase activity in these extracts, a study was conducted in collaboration with H. Y. Nakatani to look at protein degradation in one of the concentrated extracts. A portion of the pH 8.0 concentrated extract was incubated at 37°C for 24 hours after which a 3-27% gradient sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) was run using the control (extract stored frozen at -20°C) and the incubated sample. It was expected that proteolytic activity during incubation would be evident by the appearance of low molecular weight fragments in the incubated sample. Instead, it was found that a large amount of the protein in the incubated sample compared to the control was not getting into the gel. Condensation reactions between proteins or protein fragments with phenolics and other oxidation products could explain this observation.

Since endopeptidase activity in leaf extracts prepared from Nicotiana rustica had been reported by Weckenmann and Martin (1984), their published extraction and assay procedures (which were different from the procedures previously used in our laboratory) were reexamined as closely as possible using fresh, green leaf from N. rustica and Coker 319 tobaccos. Endopeptidase activity was subsequently demonstrated for both of these extracts. This activity was confirmed in several successive experiments. This was the

first time that endopeptidase activity had been repeatedly demonstrated in a tobacco extract in this laboratory without going through a concentration step. When the pH 7.0 extract and its concentrate (see above) were again tested according to the Weckenmann and Martin assay procedure, only the concentrated extract showed endopeptidase activity as had been observed before. Activity was also found when an extract was prepared according to the Weckenmann and Martin procedure from a leaf that had been frozen and stored at -80°C from the summer of 1984. Additional experiments are currently underway to optimize the assay running conditions. Furthermore, testing the substrate (azocasein) in 50 mM phosphate buffer, pH 4.5, was found to be better than testing in pH 4.5, 50 mM citrate/phosphate buffer as suggested by Weckenmann and Martin. A 2 hour incubation time for the assay was also found to give substantially higher values than a 1 hour incubation for the tobacco extracts. A time course study is planned to find the optimum incubation time for the assay with tobacco extracts. The results from a pH optimization experiment for assay running conditions showed an optimum in the range of pH 4.5 - 5.5. This experiment will be rerun to confirm these results. Experiments to study the effects of freezing on activity are also underway. Plans also include a study of the effects of certain protease inhibitors, and potential extraction components (e.g., ascorbic acid, thiourea).

Differences between leucine aminopeptidase and endopeptidase activity from extracts prepared from fresh green and fresh senesced leaf (Coker 319) have been demonstrated. When the enzyme activities were calculated on a unit of activity/hour/mg of protein basis, the leucine aminopeptidase activity was shown to decrease in the senesced leaf confirming our earlier results, whereas, endopeptidase activity was shown to increase. The latter was supported by similar results reported by Weckenmann and Martin for N. rustica.

II. Immunochemical Phytohormone Assays (In collaboration with B. Davies).

Aqueous, 80 % methanol extracts of greenhouse grown green and senesced leaf (Coker 319) were prepared for testing and methods development of an enzyme-linked, immuno-sorbant assay for abscisic acid and indole acetic acid. Transfer of these procedures to Project 1904 will occur in December, 1985.

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